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Recovery and Viability of *Edwardsiella ictaluri* from Great Blue Herons *Ardea herodias* Fed *E. ictaluri*-Infected Channel Catfish *Ictalurus punctatus* Fingerlings

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Abstract.—Feeding activities of great blue herons *Ardea herodias* in catfish ponds during outbreaks of enteric septicemia of catfish have been implicated as a mechanism for the transmission of the disease from infected to uninfected ponds. Although *Edwardsiella ictaluri*, the causative agent, has been identified in gastrointestinal tracts of great blue herons, the role of these birds as a vector of *E. ictaluri* is not well documented. The potential of these birds to contaminate catfish ponds with *E. ictaluri* was investigated by feeding captive herons over a 4-d period with catfish fingerlings injected intraperitoneally with live *E. ictaluri*. Daily fecal samples, throat and rectal swabs, and feather samples were collected, cultured and examined for *E. ictaluri* using both a selective media and a monoclonal indirect fluorescent antibody test specific for *E. ictaluri*. Gastrointestinal tracts sampled at the conclusion of the feeding trial were similarly examined. While *E. ictaluri* was detected using the indirect fluorescent antibody test, no viable *E. ictaluri* was cultured from either feces, gastrointestinal tracts or feathers. Growth of *E. ictaluri* was not observed at 40 C, the rectal temperature observed in captive great blue herons. Prior incubation at 40 C suppressed the growth of *E. ictaluri* at 24 C, an optimal temperature for growth of this bacterium. These results indicate that great blue herons appear to play little or no role in the transmission of *E. ictaluri* among catfish ponds.

Enteric Septicemia of Catfish (ESC) caused by the gram-negative bacterium *Edwardsiella ictaluri* is one of the most serious diseases encountered in the commercial production of channel catfish *Ictalurus punctatus*. A survey of catfish producers by the National Animal Health Monitoring System (NAHMS) indicated that 78% of all catfish operations and 42% of all foodfish ponds experienced outbreaks of ESC (NAHMS, USDA:APHIS 1997).

In addition to infectious diseases, commercial fish producers experience significant losses due to wildlife. The NAHMS producer survey indicated that wildlife was believed to account for 37% of catfish losses in commercial operations (NAHMS, USDA:APHIS 1997). Wildlife predation in sustained fish losses and the cost of preventing predation are estimated to cost the catfish industry $17 million annually (Wywialowski 1998). Approximately 70% of these losses are attributed to a variety of fish-eating birds including the double-crested cormorant *Phalacrocorax auritus* (53%), and the great blue heron *Ardea herodias* (42%), (Hoy 1994; Stickley et al. 1995; Glahn and Brugger 1995).

Because fish-eating birds are often observed feeding on catfish ponds during outbreaks of ESC, they have also been implicated as potential vectors in the transmission of ESC among catfish ponds and farms. A variety of bacterial pathogens of fish have been isolated from birds including *Edwardsiella tarda* from sandhill crane *Grus canadensis* (White et al. 1973); *Aeromonas hydrophila* and *Plesiomonas shigelloides* from gray heron *Ardea cinera* (Glunder 1989); and *Yersinia ruckeri* from seagulls *Larus* sp. (Willumsen 1989). Examination of gastrointestinal tracts from

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fish-eating birds collected from catfish farms also implicates these birds as vectors of ESC. Using a polyclonal fluorescent antibody procedure, Taylor (1992) detected *E. ictaluri* in 53% of the 137 birds sampled from 16 catfish ponds. Viable *E. ictaluri*, however, was recovered in only two of the samples examined during the 3-yr survey. Despite Taylor's (1992) conclusion that fish-eating birds are not major contributors to the spread or severity of ESC when other risk factors are considered, catfish producers remain concerned about the potential of birds to transmit ESC. Because the gastrointestinal tracts in the study of Taylor (1992) were taken from sporadically collected birds feeding on catfish of unknown ESC status, a more rigorous examination of the issue was suggested. The present study examined feces and gastrointestinal tracts under a "worst case scenario" in which great blue herons were fed only channel catfish fingerlings injected intraperitoneally with live *E. ictaluri*.

Materials and Methods

Great blue herons *Ardea herodias* (GBH) were trapped near Greenwood, Mississippi and transported to the National Wildlife Research Center (NWRC), Mississippi Field Station. Upon arrival, the birds were examined for physical condition, weighed, aged (juvenile or adult), wing-clipped, marked with color and number-coded patagial tags to allow for individual identification (Day et al. 1980), and released into the NWRC aviary for an observational study of feeding behavior. Following the feeding behavior study, the birds were individually housed in 9.1 m × 4.6 m × 4.6 m cages partitioned into a feeding area and fecal collection area. The feeding area contained a small plastic wading pool in which a daily ration of 340–350 g of fingerling channel catfish *Ictalurus punctatus* was provided. The daily ration was determined from the previous feeding study and studies of Schramm et al. (1987) and Stickley et al. (1995). The fecal collection area contained a perch and water dish and was lined with two layers of 3.5-mm polyethylene plastic to shield the area from accidental contamination from the heron feeding area.

After acclimating to the cages for 48 h, the birds were presented with a daily ration of *E. ictaluri* infected catfish fingerlings for each of four consecutive daily feedings. The fingerlings, taken from a raceway experiencing an outbreak of ESC, exhibited signs of chronic ESC and reacted positive for *E. ictaluri* using the ED9, anti-*E. ictaluri* monoclonal, indirect-fluorescent antibody test (ESC-IFAT) (Ainsworth et al. 1986). Immediately prior to being placed in the feeding pool, each fingerling was injected intraperitoneally with 0.1 mL of a live *E. ictaluri* suspension. Dosages of *E. ictaluri* administered to the catfish fingerlings ranged from 2.9×10^8 to 4.5×10^8 bacteria/fish with a mean of 3.3×10^8 bacteria/fish. *Edwardsiella ictaluri* used to inject fish was grown in polystyrene 25-cm^2 tissue culture flasks containing brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan, USA) for 18–24 h at 24 C on a rocker table. Following the growth period the culture was centrifuged at 600 × G for 30 min and the supernatant discarded. The pellet containing *E. ictaluri* was resuspended in Hank's balanced salt solution, pH 7.2 and the optical density at 540 nm (OD_{540}) adjusted to 1.50. The number of bacteria were estimated from a previously established curve of OD_{540} and *E. ictaluri* colony forming units (cfu). Viability and purity of the *E. ictaluri* suspensions were determined by culture on trypticase soy agar containing 5% sheep blood (BA, Becton Dickinson, Cockeysville, Maryland, USA), and a selective *E. ictaluri* medium (EIM) (Shotts and Waltman 1990). Identification used both the ESC-IFAT and the biochemical procedures outlined by Hawke (1979).

Each morning between 0800 and 0930 central standard time (CST), the individual cages were entered and the birds were captured in the fecal collection area to obtain

throat and rectal swabs and rectal temperatures. Birds were then transferred from the fecal collection area to the feeding area. While birds were in the feeding area sterile plastic spoons were used to collect and transfer feces from the polyethylene liner of the fecal collection area to sterile polyethylene sample bags. The liner was removed, the area disinfected with 0.25% solution of sodium hypochlorite from a low pressure sprayer, rinsed with a 2,200 psi pressure wash of well water, and provided with a fresh polyethylene liner.

Birds were allowed to feed on the *E. ictaluri*-infected fingerlings for a minimum of 7 h. After the feeding period, the number of fish consumed was recorded, and the birds moved again to the fecal collection area where they were held until the next morning. The same disinfection procedure was repeated each afternoon in the feeding area.

Each day the volume and weight of feces collected from each bird was recorded. The daily fecal sample from each bird was subdivided into 3 equal weight subsamples. Each subsample was diluted 1:2 with 0.22-μm filter-sterilized pond water and mixed thoroughly. A sterile 10-μL plastic inoculating loop was then inserted into each mixed subsample, stirred and withdrawn to prepare an impression smear for ESC-IFAT analysis. Additional aliquots were taken from each subsample, diluted to 0.5×10^{-5} and 0.1-mL samples used to inoculate two EIM plates and one BA plate for each fecal subsample. The dilution of 0.5×10^{-5} was determined from experiments prior to the initiation of the feeding trial to produce cfu of less than 200 total cfu/plate, the largest that could be accurately counted. Under this protocol the limit of detection for *E. ictaluri* would be 2×10^2 bacteria per sample for the ESC-IFAT and 4×10^6 per sample for the bacteriological procedure. The EIM and BA plates were incubated at 24 C and monitored for growth for 96 h. Additional fecal samples were spiked with 1.0×10^8 *E. ictaluri* for use as positive controls.

On day five of each feeding trial, birds were euthanized by carbon dioxide overdose and necropsied. Areas of the pharynx, esophagus, proventiculus, and posterior digestive tract were examined for *E. ictaluri* using both EIM media and the ESC-IFAT. Samples were vigorously swabbed (Minitip Culturettes, Becton Dickinson, Cockeysville, New York, USA), streaked on duplicate EIM plates, incubated at 24 C and examined for growth twice daily for 96 h. Duplicate samples were taken for ESC-IFAT using sterile dacron swabs (Baxter Healthcare, McGraw Park, Illinois, USA) to make impression smears from the respective areas.

Three to five feathers were taken from the thigh and abdomen of individual birds, placed in separate sterile polyethylene bags and 5 mL of sterile BHI added to each bag. The bags containing feathers and BHI broth were mixed well, incubated for 2 h at 24 C. A sterile dacron swab was then dipped into the BHI broth and used to make impression smears for ESC-IFAT. Additional ESC-IFAT examinations were performed directly on feather samples.

Bacterial colonies were counted after 72 h to 96 h incubation at 24 C. Representative colonies were screened using the DrySlide Oxidase Test® (Difco Laboratories), streaked on fresh BA plates and identified using the BBL Crystal ID System® (Becton Dickinson) and the API System (bioMérieux Vitek Inc., Hazelwood, Missouri, USA). Identification of *E. ictaluri* was based on criteria established by Hawke (1979). Composite bacterial samples were collected from each plate by streaking the plate with a sterile dacron swab and using the swab to make impression smears for ESC-IFAT. The ESC-IFAT assay was performed according to Ainsworth et al. (1986) using FITC-conjugated goat anti-mouse Ig (F2012, Sigma Chemical, St. Louis, Missouri, USA) at a dilution of 1/50 in 0.01 M phosphate-buffered-saline (PBS). Thirty to 100 fields per slide were examined at a magnification 400×. Both the number of

TABLE 1. *Sex, age, weight and rectal temperature of great blue herons* Ardea herodias *involved in the study.*

Bird ID:	Sex	Age	Weight (kg)	Rectal temperature (C)
R7	Male	Adult	2.04	39.3
R21	Female	Juvenile	2.26	40.1
Y1	Male	Juvenile	2.36	40.5
O6	Female	Adult	2.25	40.5
O15	Female	Aduk	2.45	40.5
Mean (SD)			2.27 (0.07)	40.2 (0.23)

positive fields and the total number of fields examined were recorded. Positive fields were further evaluated for bacterial morphology at magnifications of 630× and 1,000×. Positive controls for each run of ESC-IFAT slides consisted of impression smears made from BHI cultures of *E. ictaluri*.

Because the body temperature observed in great blue herons (40.2 C) exceeded the temperature stated for growth of *E. ictaluri* (Hawke 1979), the effect of 40.2 C on the viability and growth of *E. ictaluri* was examined. One-hundred microliters of a field isolate of *E. ictaluri* was used to inoculate polystyrene 25-cm² tissue culture flasks containing 10-mL BHI. Following 30 min incubation at 24 C the inoculated flasks were subjected to experimental incubation regimes. In the first experiment triplicate *E. ictaluri* inoculated flasks and an un-inoculated negative control flask were incubated at 40 C for 5 h, then returned to 24 C and incubated an additional 24 h. Similar *E. ictaluri* inoculated and un-inoculated flasks were incubated at 24 C for 29 h. At the end of the 29-h incubation period, aliquots were removed from each flask and the optical density at 540 nm (OD_{540}) determined. Additional 100-µL aliquots from each flask were plated on duplicate BA plates and examined for growth following 72 h incubation at 24 C. In experiment 2, triplicate flasks and an un-inoculated control flask were incubated at 40 C for 8 h, then incubated at 24 C for 36 h. Similar inoculated and un-inoculated flasks were incubated at 24 C for 44 h. At the end of the 44-h incubation the OD_{540} of the individual flasks was determined.

Results

The study involved three female and two male great blue herons with a mean weight of 2.27 kg (Table 1). Each bird consumed a mean of 325.8 g of ESC infected channel catfish per day representing a mean dosage of 10.1×10^9 *E. ictaluri* per day over the study period. A total of 248.2 g of feces were recovered and examined for the presence of *E. ictaluri* during the course of the study (Table 2).

The number of total bacteria recovered from feces varied with the bird, the bacterial media (BA vs. EIM) and the time of collection (Fig. 1). The number of bacteria recovered increased with time. Blood agar yielded both a significantly greater number and variety of bacteria than EIM. The dominate bacteria recovered on BA included *Edwardsiella tarda* (47.7%), *Escherichia coli* (31.8%), *Staphylococcus* sp. (13.6%) and *Plesiomonas shigelloides* (6.8%). The EIM plates appeared more selective than BA and were dominated by *E. tarda* (82.9%), *Staphylococcus* sp. (15.7%) and *E.coli* (1.4%).

A total of 107 impression smears were made from fecal samples and evaluated for the presence of *Edwardsiella ictaluri* using the ESC-IFAT. In the 8,311 microscope fields examined only 13 positive fields exhibiting both positive ESC-IFAT staining and morphology consistent with *E. ictaluri* were detected. A total of 170 impression smears were made of BA and EIM cultures

TABLE 2. *Means of daily fish consumption in weight and number of fish, dose of* E. ictaluri, *volume and weight of feces recovered for each bird. Values represent the mean of four daily feedings over the trial. Numbers in parentheses represent the standard deviation.*

Bird ID	Fish consumed		*E. ictaluri*	Feces recovered	
	Weight (g)	Number	Dosage × 10^9	Volume (mL)	Weight (g)
R7	345.4 (5.6)	37.0 (0.5)	11.9 (2.6)	9.9 (4.7)	14.6 (9.7)
R21	249.8 (41.1)	26.5 (4.5)	8.7 (2.9)	6.1 (2.7)	7.8 (2.4)
Y1	343.5 (4.2)	36.7 (0.5)	11.9 (2.7)	4.6 (0.8)	6.1 (1.4)
O6	345.2 (2.6)	27.8 (0.5)	8.9 (1.8)	5.6 (2.8)	7.8 (4.5)
O15	345.3 (2.1)	27.8 (0.5)	9.0 (2.1)	9.7 (2.7)	13.3 (4.2)
Total	6515.0 g		201.3 × 10^9 cfu		248.24 g
Mean per day	325.8		10.7 × 10^9		9.93
SD	42.4		2.7 × 10^9		5.39

from fecal samples. No ESC-IFAT positive bacteria were observed in 10,784 microscope fields (Table 3). *Edwardsiella ictaluri* was not identified from feces on either BA or EIM media using the biochemical procedures outlined above.

A total of 62 impression smears were taken from the pharynx and gastrointestinal tracts of the herons during the study. Seven ESC-IFAT bacteria were detected in the 5,270 microscope fields examined from direct impression smears of the gastrointestinal tracts (Table 4). A total of 72 impression smears were made of BA and EIM cultures obtained from gastrointestinal samples. No ESC-IFAT positive bacteria were observed in 2,360 microscope fields (Table 4). *Edwardsiella ictaluri* was not identified

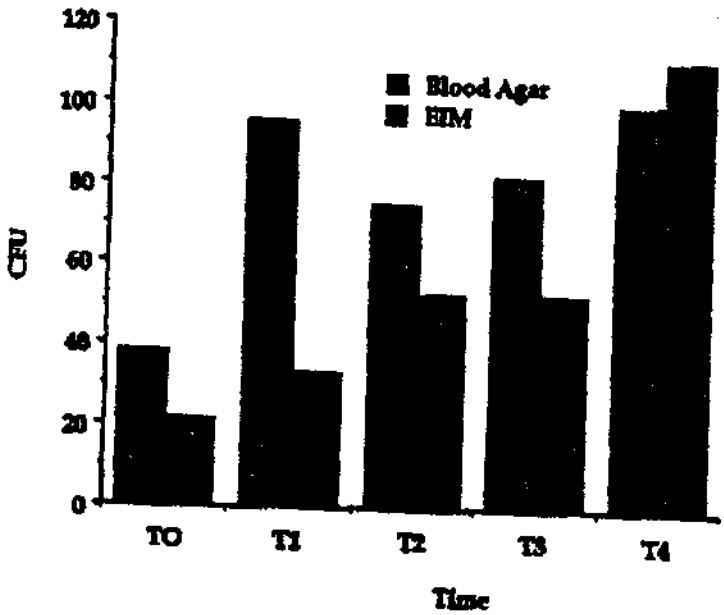

FIGURE 1. *Total bacterial colonies (CFU) cultured from great blue heron fecal samples following 10^9 dilution at respective sampling periods. The bars represent the mean CFU of fecal samples cultured on trypticase soy agar containing 5% sheep blood (Blood Agar) and a selective medium for* E. ictaluri *(EIM) (Shotts and Waltman 1990) of the five herons used in the study. Bacteria recovered on BA included* Edwardsiella tarda *(47.7%),* Escherichia coli *(31.8%),* Staphylococcus *sp. (13.6%) and* Plesiomonas shigelloides *(6.8%). The EIM plates were dominated by* E. tarda *(82.9%),* Staphylococcus *sp. (15.7%) and* E. coli *(1.4%).*

TABLE 3. *Presence of* Edwardsiella ictaluri *in impression smears and bacterial plates obtained from fecal samples of great blue herons over the 5 d of the feeding trial. The number of fields were at a magnification of 430× and the number positive represents the number of positive-staining bacteria using the ESC-IFAT assay (Ainsworth et al 1986).*

Bird ID	Fecal impression smears			Bacteriology plates		
	# Slides	# Fields	# Positive	# Slides	# Fields	# Positive
R7	15	1,334	0	45	3,900	0
R21	15	1,356	5	28	1,946	0
Y1	15	1,351	5	31	2,470	0
O6	32	2,140	0	32	1,200	0
O15	30	2,130	3	34	1,250	0
Total	107	8,311	13	170	10,766	0

from feces on either BA or EIM media using the biochemical procedures outlined above.

Edwardsiella ictaluri did not grow at 40 C. No growth was observed among BA plates or BHI cultures of *E. ictaluri* incubated at 40 C. Incubation of BHI cultures of *E. ictaluri* at 40 C for either 5 h or 8 h killed or greatly suppressed growth at 24 C, an optimal temperature for growth. (Fig. 2). No growth was observed on BA among aliquots taken from 40 C incubated BHI cultures.

Discussion

Results from the study are similar to those of Taylor (1992), who detected *E. ictaluri* in the intestines of 80% of the great blue herons sampled over a 3-yr period, but was unable to isolate viable *E. ictaluri* using either BA or EIM. During the course of the present study, great blue herons were fed a total 6.5 kg of channel catfish containing of 2.01×10^{11} *E. ictaluri*, and a total of 180 bacterial plates, 411 ESC-IFAT slides and 26,725 microscope fields were screened for *E. ictaluri*. While *E. ictaluri* was detected in feces (13 positive fields) and in the gastrointestinal tracts (7 positive fields) using the ESC-IFAT, we were unable to detect viable *E. ictaluri* in feces during the feeding trial despite the administration of a mean oral dosage of 10.7×10^9 *E. ictaluri* per day. While final dilution of the feces to 0.5×10^{-6} to obtain obtain accurate cfu counts may have limited the detection of *E. ictaluri*, no viable *E. ictaluri* was detected in direct streaks of feces or gastrointestinal tracts on either BA or EIM. *Edwardsiella tarda*, the dominate bacteria recovered in the study, readily grows at 37 C.

The gastrointestinal tract offers a harsh environment for *E. ictaluri*. The rectal temperature of great blue herons during the study, 40.2 C, (Table 1) is greater than the maximum temperature for growth of *E. ictaluri* reported by Hawke (1979). Incubation of *E. ictaluri* at 40 C suppressed both the growth and viability of *E. ictaluri*. Aliquots taken from BHI broth cultures of *E.*

TABLE 4. *Presence of* Edwardsiella ictaluri *in impression smears and bacterial plates obtained from gastrointestinal tracts and feathers of great blue herons. The number of fields were at a magnification of 430× and the number positive represents the number of positive-staining bacteria using the ESC-IFAT assay. Four pharyngeal samples were obtained for each bird prior to feeding, while two samples were obtained from each of the respective gastrointestinal areas and feathers at necropsy. No ESC-IFAT positive-staining bacteria were observed among 2,360 fields from 72 slides obtained from duplicate EIM plates obtained from each location and bird upon necropsy.*

Location	N slides (fields)	R7	R21	Y1	O6	O15	Total
Pharynx	28 (2,425)	1	1	0	2	0	4
Esophagus	8 (725)	0	0	0	0	0	0
Proventriculus	8 (725)	0	0	3	0	0	3
Intestine	9 (750)	0	0	0	0	0	0
Feather	11 (645)	0	0	0	0	0	0
Total	64 (5,270)	1	1	3	2	0	7

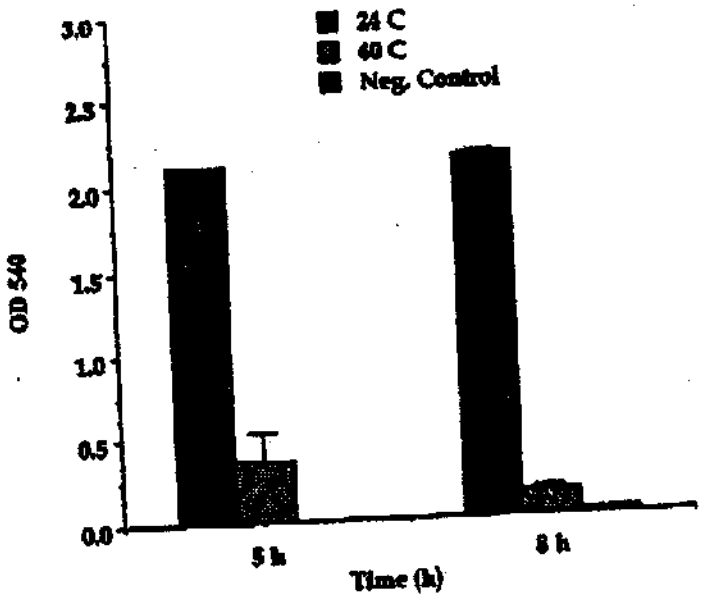

FIGURE 2. *Effect of 40 C incubation upon* Edwardsiella ictaluri. *Bars represent the mean optical density at 540 nm. Bars for 5 h represent triplicate* Edwardsiella ictaluri *cultures incubated at 40 C for periods of 5 h, then incubated at 24 C for 24 h while solid bars represent similar cultures incubated at 24 C for a total of 29 h. Bars for 8 h represent similar* E. ictaluri *cultures incubated at 40 C for 8 h then at 24 h for 36 h while solid bars represent triplicate* E. ictaluri *cultures incubated at 24 C for 44 h.*

ictaluri incubated at 40 C did not exhibit growth on BA when incubated at 24 C.

Results from this study provide little evidence that great blue herons act as vectors in the transmission of *E. ictaluri* among catfish ponds. Like Taylor, we conclude that great blue herons are not a major factor in the transmission of *E. ictaluri*. We also postulate that great blue herons may reduce transmission of *E. ictaluri* by selectively feeding on moribund fish reducing the level of infected fish in the pond.

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